THE ROLE OF HEPATIC KISSPEPTIN AS A LINK BETWEEN THE REPRODUCTIVE AXIS AND METABOLIC STATUS

by
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ABSTRACT

In mammals, the reproductive neuroendocrine axis is a feedback loop system consisting of the hypothalamus, the pituitary, and the gonads. Kisspeptin and its receptor Kiss1R (formerly, GPR54) have been established as key regulators of the reproductive axis in mammals, and mediate the neuronal output that is necessary for the preovulatory luteinizing hormone (LH) surge in females. The gonadotropin-releasing hormone (GnRH) neurons of the brain express *Kiss1r* and synapse with kisspeptin neurons. Kisspeptinergic neurons also express the estrogen receptor alpha (ERα), and are potently regulated by estrogen in a positive feedback mechanism. Previous studies have shown strong evidence of estrogen involvement in metabolic syndrome. To evaluate whether the reproductive peptide kisspeptin is affected by estrogen under altered metabolic states, we induced a 24-hour starvation in 5-6 week old male and female wild type 129SVJ mice examined whether there was a correlation between *ERα* and *Kiss1* expression in acute fasting states. We also generated a liver-specific knockout of the ERα gene in 5-6 week old male and female ERα floxed mice and measured the expression of Kisspeptin in the liver, estrous cyclicity in females, and serum gonadotropin levels. We have demonstrated that in rodents, *Kiss1* expression by the liver is highly upregulated in states of energy deficiency, while *ERα* is reduced in acute fasting states. Our data show that serum concentrations of the gonadotropin hormones LH and FSH are also affected by starvation. By virtue of its location outside the blood-brain barrier, the pituitary detects changes in levels of physiological molecules. The observations from our study provide substantial evidence for the further exploration of the reproductive response of the HPG axis under acute metabolic stress.

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INTRODUCTION

The Hypothalamic-pituitary-gonadal (HPG) Axis

The HPG axis (Fig.1) is a complex neuroendocrine feedback loop system consisting of three distinct structures. In mammals, the hypothalamus is located in the rostral forebrain, and houses the gonadotropin-releasing hormone (GnRH) - expressing neurons. GnRH neurons extend processes mainly to the organum vasculosum of the lamina terminalis (OVLT) and the median eminence (Jennes and Stumpf, 1986; King *et al.* 1982; Herde *et al*. 2011). Collectively, the OVLT and the median eminence are classified as circumventricular organs, and are characterized by their exposure to peripheral circulation, allowing for the direct regulation of these neurons by blood-borne elements influencing physiological actions such as metabolism and immunity (Herde et al. 2011). The peptide hormone from these neurons is secreted in a pulsatile manner from the median eminence, and is channeled into the hypothalamo-hypophyseal portal vasculature directly to the anterior pituitary gland, where it binds to GnRH receptors (GnRH-R) on special endocrine signal producing cells known as gonadotropes (Handa and Weiser, 2013). GnRH secretion is regulated by the interplay of neuronal input from higher cognitive and sensory centers, circulating sex steroid levels, and neuroendocrine peptides such as prolactin, activin, inhibin and leptin (Swerdloff, 2000).

In the pituitary, the ligand-receptor interaction triggers the synthesis and secretion of two distinct peptide hormones - luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH, in turn, synergistically regulate the maturation of the gonads, as well as the synthesis and secretion of the gonadal steroid hormones, namely estrogen and testosterone.

In males, the Leydig cells of the testis express LH receptors. Following the binding of LH, these cells become stimulated to produce testosterone. Sertoli cells are specialized nurse cells that support developing spermatozoa in the male gonads and express receptors for FSH (FSH-R). Previous studies have shown that mice with Sertoli cell-specific knock outs of FSH-R display a reduced testicular phenotype, implying that the role of FSH is to increase Sertoli cell, and total germ cell number (O'Shaughnessy *et al*., 2010). Testosterone imposes a negative feedback to the brain and pituitary to regulate LH and FSH secretion (O'Shaughnessy *et al*., 2010).

LH binds with high affinity to its receptors in the interstitial theca cells in the female gonads. LH ligand binding also induces signaling cascades that result in the activation of the genes in the biochemical pathway, and lead to the biosynthesis of the precursor steroid hormone androstenedione (Williams and Erickson, 2012). By means of signal transduction pathways that occur in the granulosa cells of the ovary, FSH mediates the selection and development of the dominant follicle. The dominant follicle characteristically expresses large amounts of the P450 aromatase (Cyp19) enzyme (Williams and Erickson, 2012). Aromatase activity in the granulosa cells converts the theca cell-derived androstenedione into estradiol, which then exerts feedback regulatory effects at the level of the hypothalamus and pituitary to regulate levels of LH and FSH.

Role of kisspeptin and Kiss1R in the reproductive axis

The pulsatile delivery of GnRH to the gonadotropes is critical, following evidence from previous studies that the continuous delivery of the hormone inhibits the release of LH and FSH (Belchetz, PE, 1978). Recent evidence shows that kisspeptin (Kiss1) signaling in GnRH neurons is essential and sufficient for the normal functioning of the reproductive axis, therefore making this hormone one of the principal regulators of reproduction (Novaira *et al*., 2012; Kirilov *et al*., 2013).

Kiss1 is a neuropeptide that is encoded by the *Kiss1* gene, whose product is a 145-amino acid peptide that is enzymatically cleaved into a 54-, 14-, 13-, or 10-amino acid peptides (Dungan *et al*., 2006). Kiss1 is necessary for the pulsatile release of GnRH from the hypothalamus (Keen *et al*., 2008), and evokes the release of GnRH from rat hypothalamic explants (Thompson *et al*., 2004).

The exogenous administration of kisspeptin into the brain produces a dose-dependent rise in serum levels of LH and FSH in rats (Thompson *et al*., 2004; Navarro *et al*., 2004a, 2004b; Matsui *et al*., 2004). This positive response has been reported in sheep, macaques, and humans (Messager *et al*., 2005; Shahab *et al*., 2005; Dhillo *et al*., 2005). Kisspeptin has also been shown to induce LH secretion from rat pituitary explants (Navarro *et al*., 2004b), suggesting that kisspeptin may also action directly at the level of the gonadotropes.

Anatomically, kisspeptin neurons are densely concentrated in the arcuate nucleus (Arc), which is proximal to the median eminence, and in the preoptic area, most notably, the anteroventral periventricular nucleus (AVPV), in male and female rodents (Gottsch, *et al*., 2004).

Rodents exhibit a sexual dimorphism in the expression of Kiss1 mRNA in the AVPV, as well as a subsequent response of the kisspeptin neurons in the AVPV to estradiol regulation (Dungan *et al*., 2006). In females, the expression of Kiss1 mRNA in the AVPV is more pronounced than in males. This finding is not surprising, given that the AVPV of female rodents is larger by volume with a greater number of neurons than that of male rodents (Bleier *et al*., 1982). Furthermore, the subset of neurons in the AVPV have been shown to be critical intermediates in transducing positive estradiol feedback regulating GnRH and the subsequent LH surge that is required to induce ovulation (Popolow *et al*. 1981; Wiegand *et al*. 1980; Wiegand and Terasawa; 1982, Gu and Simerly, 1997).

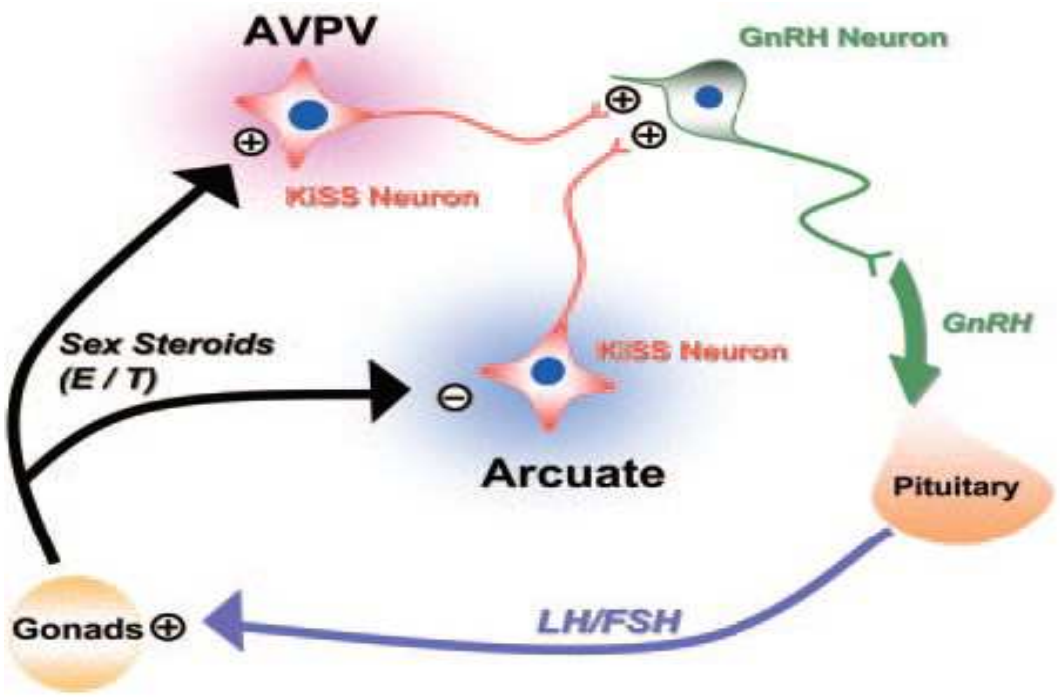


Figure 1. The HPG axis showing kisspeptin-expressing neurons in the AVPV and Arc of the hypothalamus. Sex steroids secreted from the gonads either upregulate or inhibit Kiss1 mRNA expression in the hypothalamus. Kiss1 binding to the GnRH neurons is necessary for the release of LH and FSH. (Source: Dungan *et al*., Endocrinology, March 2006, 147(3): 1154–1158)

Within the AVPV of female mice, Kiss1-expressing neurons are activated by estradiol via the estrogen receptor alpha isoform (ESR1: (Wintermantel *et al*. 2006, Smith *et al*. 2005a). In contrast, ARC Kiss1 expression is inhibited by estradiol (Smith et al. 2005a, Smith et al. 2005b), although a direct role in mediating negative feedback is not well defined.

Epigenetic regulation of Kiss1 gene expression in the brain

Rodents depend on epigenetic mechanisms to regulate estrogen in the different kisspeptin neuronal populations (Tomikawa *et al*., 2012). Histone acetylation is one such epigenetic process that regulates mammalian gene transcription. It involves the addition of A-type acetyl residues to the lysine side chains within the N-terminal region of histones by acetyltransferase (HAT) enzymes. In a non-acetylated state, the positively charged histone N-terminals interact with negatively charged DNA. However, it is hypothesized that acetyl residues diminish the binding affinity between the lysine residues and DNA (Hong *et al*., 1993; Steger and Workman, 1996) or nucleosome-nucleosome interactions (Fletcher and Hansen, 1996; Luger and Richmond, 1998), inducing a conformational change (Norton *et al*., 1989) that renders the genetic locus more accessible to the transcriptional machinery (Sterner and Berger, 2000). This phenomenon is turned off by histone deacetylases (HDACs), which return the genetic locus to a transcriptionally repressed state (Sterner and Berger, 2000).

Methylation is an epigenetic process associated with gene silencing through chromatin remodeling (Tomikawa *et al*., 2012; Hattori *et al*., 2004; Jenuwein and Allis,

2001; Tomikawa *et al*., 2006; Jones *et al*., 1981; Li *et al*., 1992). DNA methylation is the underlying molecular activity that gives rise to cellular processes such genomic imprinting, cell differentiation, embryonic development, X-chromosome inactivation and chromosome structure stability (Phillips, 2008). Mammalian genomic DNA is methylated at cytosine residues at CG dinucleotide (CpG) sequence sites linked by phosphate groups (Gruenbaum *et al*., 1981). In the "*de novo*" model of methylation, DNA methyltransferase (DNMT) enzymes catalyze the transfer of the methyl group from S-adenosyl methionine (a naturally occurring cellular compound derived from the breakdown of ATP) to the cytosine bases to form 5-methylcytosine. In their role as "maintenance" DNMTs, the enzymes copy existing methyl sequences attached in the *de novo* pathway onto daughter strands following DNA replication (Phillips, 2008). The presence of methyl groups near promoter regions has been hypothesized to block the binding of transcription factors, and correlate with the observation of little or no transcription at DNA methylated sites (Suzuki and Bird, 2008; Phillips, 2008).

Tomikawa and colleagues (2012) have investigated the association between estrogen and histone acetylation and DNA methylation in the *Kiss1* gene locus of murine AVPV and Arc nuclei. In their experiment, the researchers administered exogenous estradiol to harvested brain tissues of ovariectomized (OVX) mice and observed a significant increase in H3 acetylation at the *Kiss1* promoter region in the AVPV. In contrast, the Arc showed a significant reduction in H3 acetylation in the same promoter region in response to estrogen. The authors further validated that ERα recruitment to the *Kiss1* promoter region was enhanced in the presence of estrogen in the AVPV but not in the Arc. It was also noted that the CpGs upstream of the transcriptional start sites of

Kiss1 expressing neurons showed hypermethylation, with no significant difference between *Kiss1*-expressing cells (i.e. AVPV, Arc, differentiated trophoblast stem cells) and non-*Kiss1*-expressing cells. Using a chromatin conformation capture (3C) assay, the study demonstrated a chromatin loop formation between the promoter and the 3' region in the *Kiss1* locus of the AVPV when estrogen was present, suggesting that the 3' region "enhances the induction of estrogen-dependent AVPV *Kiss1* promoter activity." Although loop formation was noted in the Arc following estrogen treatment, the pattern was different from that observed in the 3' enhancer region of the *Kiss1* locus of the AVPV, and this was hypothesized to imply that the 3' region mediates the estrogen suppression mechanism of *Kiss1* expression in the Arc.

Overall, this study shows that in the presence of estrogen, ERα binds to the promoter region of *Kiss1*, where it induces epigenetic changes leading to the H3 acetylation as well as chromatin loop formation that results in AVPV *Kiss1* expression. Conversely, in the Arc estrogen exhibits the opposite epigenetic regulation that gives rise to the reduced expression of *Kiss1*.

Estrogen receptors and estrogen signaling

The primary estrogenic hormone 17β-estradiol (E_2) is synthesized in the ovary and in other tissues by androgen aromatization (Marino *et al*., 2006). In the ovary, the conversion of androstenedione to estradiol is catalyzed by Cyp19 (aromatase), which is regulated by FSH action in the granulosa cells (Hillier *et al*. 1981, Ryan, 1979). E_2 plays a wide role in mammalian physiology, modulating the regulation of reproductive,

metabolic, inflammatory, neurological and cardiovascular functions (Musatov *et al*., 2007; Gruber *et al*., 2002; Pearce *et al*., 2004; Deroo *et al*., 2006).

E_2 mediates its biological effects through its intracellular receptor isoforms – alpha (ESR1 or ERα) and beta (ESR2 or ERβ) – that are both encoded by distinct genes located on different chromosomes (Hall *et al*., 2001). In vitro (Couse *et al*., 1997) and in vivo (Barros and Gustafsson, 2011) studies have shown that ERα and ERβ play different roles, and are localized to different tissues. ERα is predominantly expressed in the breast, uterus, cervix, vagina, whereas ovarian, prostate, testicular, splenic, pulmonary, hypothalamic and thymic tissues sparsely express ERβ (Hall *et al*., 2001). Global ERα knockout studies give rise to mouse phenotypes with hypoplastic male and female reproductive tissue, hypergonadotropic hypergonadism, undeveloped mammary glands, excess fatty tissue, and infertility (Couse and Korach, 1999; Hall *et al*., 2001). Conversely, ERβ mouse models display superficially normal phenotypes although fertility - as a function of litter size - is reduced, and females exhibit progressive ovarian germ cell loss with re-differentiation of the surrounding somatic cells (Couse *et al*., 1999; Dupont *et al*., 2000; Hall *et al*., 2001).

The general structure of ERα and ERβ, both belonging to the nuclear receptor superfamily of proteins, is shown in Figure 2 below. Although the DNA- and ligand-binding domains of ERα and ERβ are highly conserved, there is substantial divergence at the N-terminus (Hall *et al*., 2001). It is this N-terminus that has been found to play a role in intermolecular and intramolecular interactions, and in gene regulatory activities (Marino *et al*., 2006). The two zinc finger structures (not shown) of both receptors enable them to bind with high affinity to specific, inverted palindromic sequences (Klinge, CM,

2001; Marino *et al*., 2006) known as estrogen response elements (EREs). Whereas the hinge domain (Fig.2, D region) allows for receptor dimerization and binding to chaperone heat-shock proteins, the ligand-binding domain (LBD, E/F region), in association with the N-terminus, modulates ER function in gene transcriptional activity (Marino *et al*., 2006; Nilsson *et al*., 2001; Mosselman *et al*., 1996; Claessens and Gewirth, 2004; Kumar *et al*., 2004).

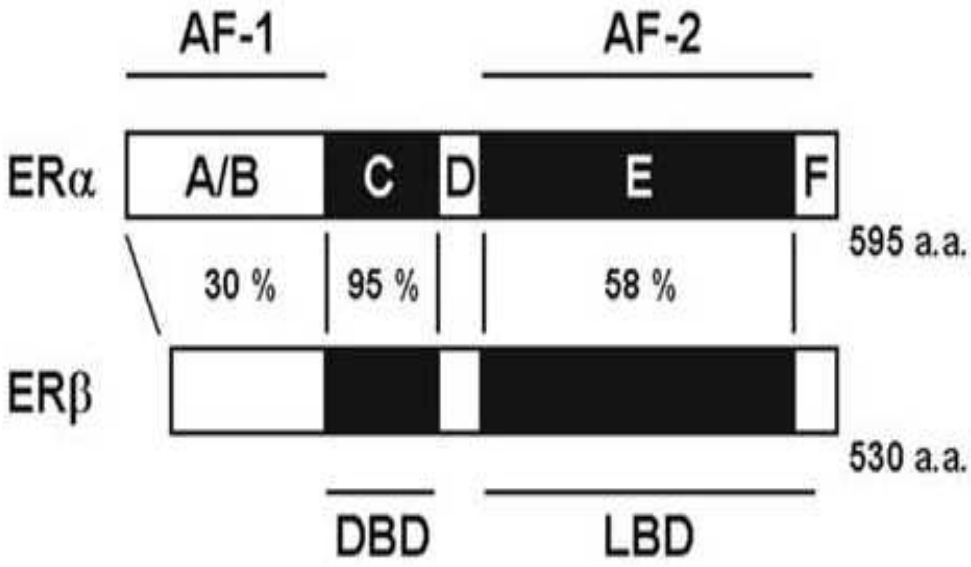


Fig. 2 The molecular domain structure of human ERα and ERβ. Both receptor isoforms consist of the N-terminal domain (A/B) involved in transactivation (AF-1 and AF-2). The ligand-binding domain (LBD, E/F region) binds E2 to the receptor, and the hinge region allows for receptor dimerization and bindging to hsp. (Source: Marino, M. et al., Current Genomics, 2006, Vol. 7, No. 8)

Two activation function domains - AF-1, located in the N-terminus (A/B), and AF-2, embedded within the ligand-binding domain (LBD, E region) - characterize the ER structure. Depending on the cell-specific context, AF-1 and AF-2 interact with transcription co-activators and regulate transcriptional activity either independently or in synergy with each other (Marino *et al*., 2006; McEwan, IJ, 2004).

Ligand-activated ERs must interact with transcriptional cofactors (either co-activators or co-repressors) in order to affect transcriptional activity (McKenna *et al*., 1999). Cofactors serve as the platform for assembling other proteins involved in transcriptional regulatory activities. The most characterized ER cofactors are the members of the steroid receptor co-activator family of proteins (SRC-1, 2 and 3) that interact with the amphipathic α-helix domain of AF-2 (McKenna *et al*., 1999; Webb *et al*., 1998). Others include the thyroid hormone receptor associated protein/ vitamin D receptor-integrating protein (TRAP/DRIP) complex, the histone acetyl transferase (e.g. CBP/p300), histone methyl transferase (e.g. CARM1 and PRMT1), and the nucleosome remodeling (e.g. SWI, SNF) complexes (Smith and O'Malley, 2004).

On some genes, ER has been shown to associate with special promoters and enhancers including activating transcription factor-2 (ATF-2) /c-jun or the ATF-2/cAMP response element binding protein (CREB) (Marino *et al*., 2006; O'Lone *et al*., 2004).

In the classical ligand-dependent signaling pathway, E_2 diffuses from the blood vessels through the plasma membrane of target cells, where it binds to either of one its two receptor isoforms and forms a nuclear-receptor complex. When there is no hormone present, the steroid nuclear receptors remain in a multi-protein inhibitory complex in the target cell nucleus; however, in the presence of the E_2 ligand they undergo a conformational change within the ER with resultant high affinity binding to EREs of target genes where, as a transcription factor complex, E_2–ER-ERE plays a role in gene silencing or upregulation (Hall *et al*., 2001; McKenna *et al*., 1999). Interestingly, in vitro studies have shown that when ESR1 and ESR2 are co-expressed they form heterodimers, suggesting that there may be a signaling pathway convergence between the two receptors

(Hall *et al*., 2001).

E_2 signaling leads to the activation of four main signaling pathways: 1. phospholipase C (PLC)/protein kinase C (PKC) (Morley *et al*., 1992; Marino *et al*., 1998; Marino *et al*., 2001a; Marino *et al*., 2001b; Picotto *et al*., 1999; Perret *et al*., 2001; Incerpi *et al*., 2003), 2. Ras/Raf/MAPK (Marino *et al*., 2002; Watters *et al*., 1997; Russell *et al*., 2000; Dos Santos *et al*., 2002; Migliaccio *et al*., 2002; Tanaka *et al*., 2003; Klinge *et al*., 2005; Woo *et al*., 2005), 3. phosphatidyl inositol 3 kinase (PI3K)/AKT (Björnström and Sjöberg, 2005; Levin, ER, 2005; Ascenzi *et al*., 2006; Marino *et al*., 2003; Acconcia *et al*., 2005; Marino *et al*., 2005, Castoria *et al*., 1999; Castoria *et al*., 2001; Chambliss *et al*., 2005; Simoncini *et al*., 2000; Alexaki *et al*., 2006), and 4. the cyclic AMP (cAMP)/ protein kinase A (PKA) pathways (Picotto et al., 1999; Farhat *et al*., 1996). Other signaling mechanisms occur through "transcriptional crosstalk," i.e. without the binding of ER to ERE sequences. These indirect signaling pathways include the interaction between ERα and the c-*rel* subunit of the nuclear factor – κB (NF- κB) complex that inhibits the expression of the inflammatory cytokine interleukin-6 (IL-6) (Marino et al., 2006; Galien and Garcia, 1997).

Estrogen and metabolic functioning, and the role of hepatic ERα

Bailey and Ahmed-Sorour (1980) examined the link between ovarian hormones and the long-term control of glucose homeostasis. These investigators found that compared to wild type, OVX mice had a 40% increase in plasma glucose concentration levels during glucose tolerance tests, a 26% decrease in plasma insulin response to glucose, and a 32% decrease in plasma insulin responses to arginine. Histology examinations from the same

study revealed that pancreatic and islet tissue volume decrease by 36% and beta cell number is reduced by 39% in OVX mice. The reintroduction of estrogen to OVX mice has been shown to reverse these defects (Bailey and Ahmed-Sorour, 1980; Lindberg *et al*., 2002. Clinically, postmenopausal women who undergo hormone replacement therapy with E_2 report lowered visceral adipose tissue, fasting serum glucose and insulin levels (Matic *et al*., 2013; Munoz, J *et al*., 2002).

E_2 signaling, through ERα, is linked with energy homeostasis and metabolic defects (Musatov *et al*., 2006; Matic *et al*., 2013). Whole body knockouts of ERα in mouse studies result in elevated insulin levels, impaired glucose tolerance, and increased visceral fat (Musatov *et al*., 2006). Interestingly, ERβ global knockout mice do not display altered insulin responses or abnormal weight-gain, suggesting that of the two receptor isoforms ERα plays a more direct role in metabolic functioning.

The ventromedial nucleus (VMN) consists of a high density of E_2 binding sites with high neuronal expression of ERα (Musatov *et al*., 2006). Targeted ablations of ERα in the VMN give rise to an obese phenotype, albeit with marked hyperphagia (Musatov *et al*., 2006). In the case of the VMN ERα knockouts, fat accumulation was most notable in visceral but not subcutaneous depots. This finding is crucial, because although obesity describes the accumulation of adipose tissue in either or both regions, visceral adiposity is strongly associated with the hallmark features of metabolic defects (Wajchenberg, BL, 2000; Musatov *et al*., 2006).

The liver is a major site of metabolic activity in mammals. Estrogen regulation via ERα has also been shown to play an important role in hepatic function. Aromatase deficient mice exhibit impaired hepatic function, which is reversible following E_2

therapy. Disruption of the *ERα* gene in the liver has produced conflicting results (Zhu *et al*., 2013; Della Torre *et al*., 2011; Matic *et al*., 2013) in genetically modified mouse models. While one group identifies a role for hepatic *Esr1* in regulating IGF1 synthesis, puberty and growth (Della Torre et al., 2011), another group reports no difference in metabolic function, growth or fertility, and a third has shown that hepatic ERα was required to prevent the development of fatty liver and impaired hepatic insulin sensitivity in mice fed HFD (Zhu *et al*., 2013).

In sum, the above studies demonstrate the interrelationship between the estrogenic pathway and the metabolic signaling of mammals. Metabolic syndrome is characterized by the display of abnormalities such as hypertension, dyslipidemia, type II diabetes, and obesity. Estrogen has been shown to play a protective role in modulating the effects of these pathologies in experimental animal models, such as in OVX mice, and clinically, in hormonal therapies for postmenopausal women.

Kisspeptin role in metabolic functioning and reproduction

Most studies exploring KISS1 regulation of reproduction have focused primarily on actions in the hypothalamus, but a potential role for KISS1/KISS1R at the level of the pituitary or placenta, where the *Kiss1R* is also highly expressed, has been proposed. While there is little evidence for direct activation by hypothalamic KISS1, pituitary KISS1R could mediate effects of circulating KISS1, either by directly regulating gonadotroph secretion, or by interacting synergistically or modulatory with other signals such as GnRH, insulin or E_2.

Although the action of non-hypothalamic Kiss1 on the HPG axis has been unexplored, the pituitary, by virtue of its location outside of the blood brain barrier, is poised to serve as an integrative sensor for peripheral metabolic status, peripheral reproductive status, and central signals regulating reproduction.

Changes in metabolic states, occurring mainly through fasting and feeding, can profoundly affect the reproductive axis (Dungan *et al*., 2006). Fasting results in reduced gonadotropin levels and anovulation (Dungan *et al*., 2006). Castellano and coworkers (2006) noted a reduction in neuronal *Kiss1* mRNA expression with delayed pubertal onset (as measured by vaginal opening) in chronically sub-nourished female rats compared with control rats fed *ad libitum*.

Food availability is communicated to the hypothalamus by leptin, which is a cytokine secreted by white adipose tissue. Obese (*ob*/*ob*) mouse models with impaired leptin signaling also give rise to reduced hypothalamic *Kiss1* mRNA expression, with rescue of the latter phenotype occurring by exogenous leptin administration (Foukas *et al*., 2006; Quennell *et al*., 2011).

Our group has observed relatively high levels of *Kiss1* mRNA in the livers of both male and female mice. Since estrogen regulation of hypothalamic *Kiss1* plays an important role in mediating estrogen feedback regulation of the HPG axis, we further propose a role for hepatic E_2 in regulating hepatic *Kiss1*, which may serve as a communication hub between the reproductive axis and metabolic function.

Previous studies from our lab noted evidence of a high upregulation of the *Kiss1* gene in a mouse model with constitutive hepatic activation of PKA signaling (L-Δ-prkar1a

mice). These mice exhibit hyperglycemia suggesting that insulin secretion was insufficient to respond to the increased glucose levels (Hussain *et al*., 2014 in press).

Hypothesis

We therefore hypothesize that since the liver is a key organ regulating metabolic function, ERα and Kiss1 expression are affected by changes in metabolic states, and the liver is source of circulating Kiss1, then hepatic Kiss1 integrates metabolic signals to regulate the central reproductive axis.

EXPERIMENTAL METHODS

To test our hypothesis, we first assessed whether hepatic kiss1 mRNA expression is affected by changes in metabolic states. We have investigated the above relationship through the following experimental methods:

1) Determined whether caloric restriction affects the expression of ERα and Kiss1 in the liver;

2) Determined the effect of caloric restriction on serum gonadotropin levels;

3) Investigated *Kiss1* mRNA expression upon a knockdown of liver -specific ERα;

4) Determined the changes in reproductive phenotype following hepatic ERα knockdown, and

5) Determined the changes in metabolic phenotype following hepatic ERα knockdown

MATERIALS AND METHODS

Animals

Five-week old wild type (WT) male and female 129/SvJ mice used in Experiments 1 and 2 were purchased from The Jackson Laboratory (Bar Harbor, ME).

Floxed ERα mice used in Experiments 3, 4, and 5 were bred in the animal facility of the Johns Hopkins School of Medicine Miller Research Building (Baltimore, MD). Floxed ERα mouse lines were generated by inserting 34-bp loxP sites on either side of exon 3 of the ERα gene. Exon 3 encodes the DBD and is important for ER-ERE binding, as well as the transcriptional regulation of ERα target genes.

Briefly, this method involved the insertion of a self-excising neomycin cassette consisting of the testis-specific angiotensin-converting enzyme (ACE) promoter driving the expression of *Cre* recombinase (tACE-Cre/Neo) into exon 3 of the gene. Insertion was made by electroporation into 129/SvJ mouse embryonic stem (ES) cells at the Johns Hopkins University Embryonic Stem Cell Core. Males heterozygous for the integrated recombinant were mated with wild type C57BL/6 females, giving rise to offspring with a deletion of the tACE-Cre/Neo cassette, with the exon 3 flanked by two loxP sites (Chen *et al*., 2009). Genomic DNA genotyping from tail snip isolations was carried out by polymerase chain reaction (PCR) to confirm the presence of the floxed allele. The primer pair sequences P1: 5'-AGGCTTTGTCTCGCTTTCC-3', P2: 5' -GATCATTCAGAGAGACAAGAGGAACC-3' were used to amplify 881bp and 741bp band sizes corresponding to the floxed ERα and wild type ERα fragments, respectively. All experimental procedures were reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

Tissue harvest and RNA isolation

300mg of liver tissue was harvested and stored overnight at -70ºC in 1.5-ml Eppendorf centrifuge tubes. The tubes containing the samples were placed ice, and 1ml of TRIzol reagent (Invitrogen, Carlsbad, CA) was added to each tube, and mechanically homogenized. Homogenized samples were incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complex. 0.2ml of chloroform was then added and the tubes were vigorously shaken by hand for 15 seconds. After incubating again at room temperature for 3 minutes, samples were centrifuged at 12,000 x *g* for 15 minutes at 4ºC. A phase separation was observed with the colorless upper aqueous phase containing the RNA. This phase was removed by angling the tubes at 45º and pipetting out the solution into newly labeled 1.5ml tubes. The total RNA precipitation procedure began with the addition of 500µl of isopropanol, followed by incubation at room temperature for 10 minutes, and centrifugation at 12,000 x *g* for 10 minutes at 4ºC. The supernatant was removed from the tube, leaving the visible RNA pellet, which was washed with 500µl of 70% ethanol. The sample was vortexed briefly and centrifuged at 7500 x *g* for 5 minutes at 4ºC. The wash was discarded and the RNA pellets were allowed to air dry for approximately 10 minutes. Extracted RNA was resuspended in RNAse free water, sample RNA yields were determined by UV spectroscopy, and stored at -70ºC until further analysis.

RT- quantitative PCR

Real-time quantitative PCR (RT-qPCR) is a high-throughput, sensitive assay that allows for the detection of gene amplification in real time. RT-qPCR compares the relative expression level between an internal reference gene, i.e. one that exhibits constant expression levels in all cell types, and the gene of interest. This technology makes use of fluorogenic probes (reporter dyes) that hybridize to the amplicons. In the extension phase of the amplification reaction, the nucleolytic activity of the *Taq* polymerase cleaves the probe in a process that is accompanied by fluorescent emission. Fluorescent emission spectra are measured in real time by charged-couple device (CCD) cameras, and are analyzed by computer software (Heid *et al*., 1996; Gibson *et al*., 1996). The software algorithm yields a calculation of the cycle at which the amplicon reaches an arbitrary level of 10 standard deviations above the baseline levels. This variable, known as the comparative threshold (C_T) value is essentially a "quantitative measurement of the copies of the target found in any sample" (Heid *et al*., 1996; Gibson *et al*., 1996).

Our real time-PCR experiments were performed in triplicates in 96-well plates. We ensured that C_T values for each triplicate showed minimal variation. As expected, raw data showed linearly decreasing C_T values with increasing target gene quantity.

One μg of extracted RNA was subjected to reverse transcription using an iScript cDNA kit (Bio-Rad Laboratories, Hercules, CA). Taqman qPCR was performed to detect relative ERα mRNA expression levels, with the housekeeping gene GAPDH serving as an internal control (reference gene) for cDNA input. SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA) was used in *Kiss1* detection assays with the iCycler qPCR machine (Bio-Rad Laboratories). For the Taqman qPCR assay, we utilized the

following primer pair sequences: GAPDH sense 5'-GGGCATCTTGGGCTACACT-3' and antisense 5'GGCATCGAAGGTGGAAGAGT-3' with the hybridization probe 5'-AGGACCAGGTTGTCTCCTGCGA-3' labeled with Cal fluoro red-610 and BHQ-2. ERα-specific primer sequences consisted of sense strand 5'-GGTGCCCTACTACCTGGAG-3' and antisense strand 5'-GCCCACTTCGT AACACTTGCG C-3'. The primers used to amplify Kiss1 were sense primer, 5'_-AGCTGCTGCTTCTCCTCTGT-3', and antisense primer, 5'_GGACTGCTGGCCTGTGGAT-3'. Real-time qPCR was performed under the following cycling conditions: 94ºC for 3 min, 40 cycles of 94ºC for 30 s, 60ºC for 30s, and 72ºC for 30s on an iCycler iQMulti-color real-time PCR detection system (Biorad, Hercules, CA).

We used the $\Delta\Delta C_T$ mathematical formula C_T (experimental) - C_T (control) in our analysis to obtain differences between control and experimental animal gene of interest expression. mRNA levels were calculated as fold difference from the average of the control samples using fold change = $2^{\Delta\Delta}C_T$).

Effect of caloric restriction on hepatic Kiss1r and ERa mRNA expression

This experiment involved 4 groups each consisting of 5 five week-old male and female WT 129/SvJ mice that were subjected to different feeding conditions over a 24-hour experimental period. Experimentation was carried out separately for male and female mice, making up a total of 40 mice used in our analysis.

Group A consisted of control mice that were fed *ad libitum* over the 24-hour

period. Animals in **Groups B**, **C** and **D** were fasted for 24 hours, but supplied with water during the experimental period. Group B animals (also referred to as the "complete fast" group) were sacrificed and tissues were harvested without the reintroduction of chow after 24 hours. We sacrificed and harvested tissue from Group C and Group D mice at 1 hour and 4 hours, respectively post-fasting following the reintroduction of chow. Liver tissue samples were harvested for determination of relative hepatic ERα and Kiss1r mRNA expression levels by RT-qPCR as described above.

Effect of caloric restriction on serum gonadotropin levels

Serum from blood samples was used to measure LH and FSH levels from the 4 experimental groups. For females, the 24-hour caloric restriction and re-feeding timeframe was set up to end just before 10:00AM, at a period that circumvented the LH surges of the proestrus cycle. Blood samples were collected from mandibular venous plexuses and centrifuged at 7,500 x*g* at 4^{o}C for 15 minutes to separate the serum from red blood cells (RBCs). RBCs were discarded, and the serum was collected and stored at -70^{o}C until further analysis.

Gonadotropin levels were measured using a Milliplex MAP immunoassay (Mouse Pituitary panel; Millipore, USA) on a Luminex 200IS platform (Luminex Corporation). The detection limit for LH was 0.012 ng/mL and for FSH was 0.061 ng/mL. A standard curve was generated using 5-fold serial dilutions of the manufacturer's reference hormone.

Kiss1 mRNA expression following liver-specific knockdown of ERα

Male and female ERα floxed mice were injected with an adeno-associated virus vector expressing the *Cre* recombinase under the control of the liver-specific thyroxine-binding globulin (TBG) promoter (AAV8.TBG.PI.Cre.rBG) in the tail vein to selectively disrupt ERα. Control mice were injected with AAV8.TBG.PI.eGFP.WPRE.bGH, which did not include the *Cre* recombinase. Virus vectors were obtained from the University of Pennsylvania School of Medicine Vector Core (Philadelphia, PA). The *Cre* recombinase catalyzes the recombination of the unidirectional loxP sequences flanking the ERα gene, and in a loop formation fashion, excises this gene from hepatocytes, thereby inducing a knockout.

We injected $1x10^{11}$/ml of either Cre or control virus vectors diluted to a final volume of 100µl with sterile phosphate-buffered saline into a total of 12 mice (6 males; 6 females - 3 Cre; 3 control, each) with a 30-gauge needle fixed to a 1-ml syringe. After 14 days, mice were euthanized, and liver tissues were harvested for analysis of mRNA expression of *ERα* and *Kiss1* by quantitative real-time PCR.

Reproductive phenotype assessment

Puberty and estrous cyclicity were analyzed in 6 week-old female floxed ERα mice daily for 10 days after day 3 of the tail vein injections of the Cre virus vector. Vaginal smears were collected every morning before 10:00AM. 10µl of PBS was expelled into the vagina and aspirated back into the tip of the 10µl pipette tip and dropped into the center of a frosted edge microscope slide (Thermo Fisher Scientific, Waltham,

MA). Smears were made by circling the vaginal fluid onto a 1.5mm diameter area in the center of the slide. Slides were allowed to air dry and fixed in absolute MeOH for 30 seconds, and drained and stained with the Diff-Quick stain kit (IMEC, Inc.).

Table 1 below shows the description estrous cycle stages as described by Nelson *et al*. (1982). The cycle sequence begins with proestrus, in which the individual stages were classified as diestrus/proestrus (DP) if we observed a thin smear density with high leukocyte to epithelial cell ratio, proestrus (P) if there was a medium smear density with predominantly well-formed nucleated epithelia, proestrus/estrus (PE) with medium smear density and high amounts of cornified epithelia and nucleated epithelia. The estrus (E) cycle stage marks ovulation, and is accompanied by a distinct show of cornified epithelia. We observe high amounts of leukocytes, nucleated and cornified epithelia in the metestrus 1 and 2 (M1 & M2) stages, and the diestrus (D) stage is marked by predominantly leukocytic cells on a thin smear density.

TABLE 1. Classification of stages of the estrous cycle by cell morphology in vaginal smears.

Stage of cycle	Cell type[a]			Smear density
	Leukocytes	Nucleated epithelia	Cornified epithelia	
Diestrus/proestrus (DP)	+ to ++[a] (Predominant)	+ Well-formed	0 to +	Thin
Proestrus (P)	0 to + Often degenerating	+ to +++ Well-formed (predominant)	0 to +	Medium
Proestrus/estrus (PE)	0	+ to ++	++ to +++ (Predominant)	Medium
Estrus (E)	0	0	++ to +++ Relatively small cells (predominant)	Medium to heavy
Metestrus 1 (M1)	0 to ++	0	++ to +++ Larger, more flat and clumped than in estrus (predominant)	Medium to heavy
Metestrus 2 (M2)	++ to +++ (Predominant)	+ to ++ Often irregularly shaped and vacuolated	+ to ++	Medium to heavy
Diestrus (D)	+ to +++ (Predominant)	+ Often irregularly shaped and vacuolated	0	Thin

[a]Cell density: 0=none, +=few, ++=moderate, +++=heavy.

Table 1. **Reproductive phenotype assessment through estrous cyclicity determination**. Microscopic examinations of vaginal smears show different cell types corresponding to different stages of the estrous cycle. (Source, Nelson *et al*., 1982. *Biol. of Repro*., **27**, 327-339). BIOLOGY OF REPRODUCTION 27, 327-3 39

In this study, we defined cycle regularity as the frequency of estrus, as well as the ratio of the frequency of contiguous 4-day frequencies spent in the different phases between control and hepatic ERα knockdown mice. We defined a normal estrous cycle as the observation of leukocytic (L) vaginal cytology for 2 d followed by 1 d of nucleated (N), and 1–2 d of cornified (C) vaginal cytology.

Metabolic phenotype assessment through glucose tolerance tests

Glucose tolerance tests (GTT) were performed to measure the changes in blood glucose levels following the administration of glucose. GTT were performed after fasting both experimental and control mice overnight (16 hours) and injecting them intraperitoneally (IP) with 2g/kg body weight of dextrose. Blood samples were collected from the tail vein by cutting 2mm of tissue from the tail tip distal to the bone with sharp razor blades, and obtaining blood by massaging the tail ("milking") and collecting blood into capillary tubes and placing a drop onto glucometer strips at baseline level (T = 0) before the injection of glucose, and at 15-, 30-, 90-, and 120- minute intervals following IP glucose injections (Ayala *et al*., 2010). Blood glucose levels were measured with a One Touch Ultra Blood Glucose Meter (LifeScan, Inc.).

Statistical Analyses

All data were analyzed and graphed with the GraphPad Prism 4 Program (GraphPad Software, Inc., San Diego, CA). Values are expressed as mean ± S.E.M. and different letters represent statistically significant differences between groups. Unpaired student's t-tests were used in analyzing differences of significance between control and experimental groups. Two-way ANOVA was used to analyze differences in LH and FSH levels between control and experimental groups. Statistical significance was set at an alpha level below .05 ($p<0.05$).

RESULTS

1) Effect of caloric restriction on hepatic Kiss1 and ERα mRNA expression

A B

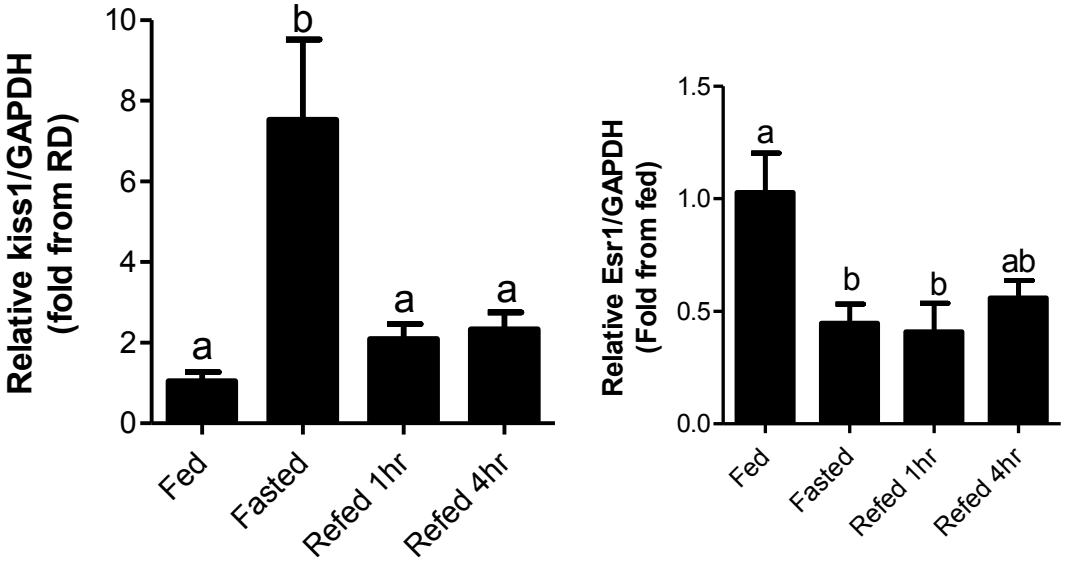


Figure 3. Quantitative PCR results of hepatic expression levels of (A) Kiss1 mRNA and (B) ERα mRNA relative to GAPDH mRNA expression after 24 hour fasting in male and female control (fed) and experimental groups. In (A), kisspeptin expression is strongly upregulated in fasted states ($p<0.05$), and in (B), ERα is elevated in with continuous feeding ($p<0.05$). Graphs show relative Kiss1/GAPDH fold expression (**A**) relative to complete restricted diet (RD) and ESR1/GAPDH expression (**B**) relative to control fed groups. Letters **a** and **b** represent statistical significant difference between groups.

We assessed the effect of caloric restriction and feeding on the expression of hepatic *ERα* and *Kiss1*. Control mice (Group A) were fed *ad libitum* over a 24-hour period, while 3 experimental groups underwent fasting over the same time frame. Group B of the experimental cohort was sacrificed and tissues were harvested for analysis after

the 24-hour fasting period, whilst Groups C and D were sacrificed 1-hour and 4-hours after the reintroduction of feed following the 24-hour fasting period.

Figure 3a shows that in the fasted state, Kiss1 expression is elevated in the liver ($p<0.05$), showing an approximately 6-fold difference between the control and fasted states. One hour after reintroducing chow, mRNA expression level reduces significantly, and further attenuates to the baseline level as observed in the control group.

From **Figure 3b** it is evident that there is a relatively high expression of the *ER*α mRNA under fed state. When mice are fasted, the mRNA levels of this receptor are significantly reduced ($p<0.05$). We note that expression remains low even 1 hour after introducing feed, and begins to approach baseline levels after 4 hours post-feeding.

Taken side by side, our data show that hepatic *Kiss1* and *ER*α are both regulated by metabolic status. Furthermore, we observe that the expressions of Kiss1 and ERα in the liver are inversely correlated to each other, where in the fed states liver-specific *ER*α is robustly upregulated and *Kiss1* expression is low; however, in the fasted state, *ER*α levels are significantly reduced, while *Kiss1* levels are markedly increased.

2) Caloric restriction and serum gonadotropin levels

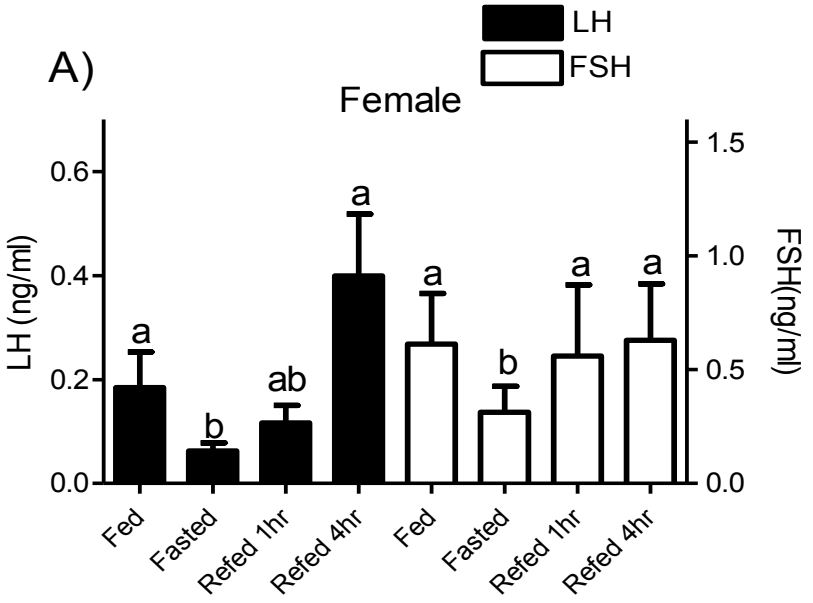


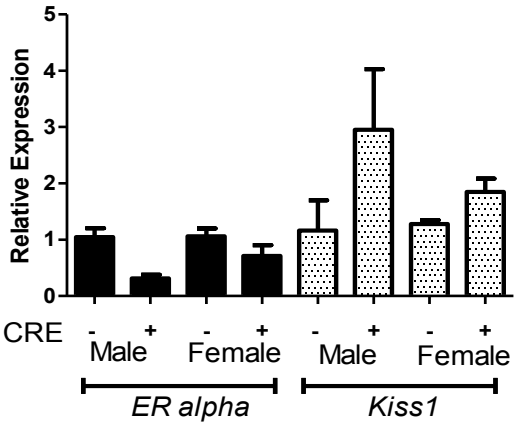
Figure 4. **Serum levels of LH (closed bars) and FSH (open bars)** in ng/ml in the control group, normally fed over the 24-hour experimental period, the completely fasted group, and groups with serum level assessments performed 1 hour and 4 hours following the reintroduction of feed. Student's unpaired t-test performed for control and experimental values, with α value for significance set at 0.05. Data are shown as mean ± standard deviation.

Blood samples from the mandibular venous plexus were obtained from our 4-cohorts of males and female mice, consisting of control animals fed *ad libitum* over 24 hours, and 3 fasting groups housed without feed for 24-hours. Serum gonadotropin levels were assessed with the Milliplex MAP bioimmunoassay kit. Serum LH levels significantly reduce in the fasted state (0.08±0.05) ng/ml, compared to when mice were fed normally (0.18±0.1) ng/ml. One hour after feeding, following 24 hours of caloric restriction, LH levels began to significantly increase, and reach notably high levels at 4-hours post-feeding.

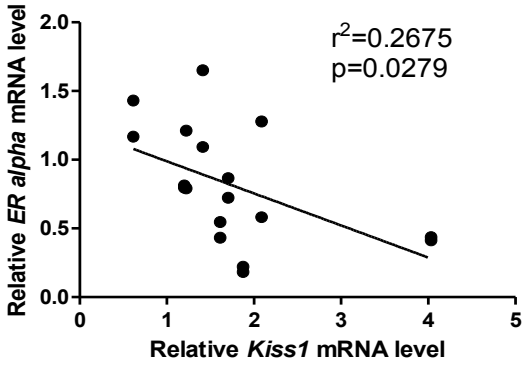
Under caloric restriction, FSH levels are lowered significantly (0.6± 0.07 ng/ml) relative to when they are normally fed. At 1 and 4- hours post-feeding, serum samples taken from the experimental group that had food reintroduced following the 24-hour fast showed statistically significant ($p<0.05$) increase in FSH levels similar to baseline levels.

3) Liver-specific knockdown of ERα in floxed mice

A)



B)



C)

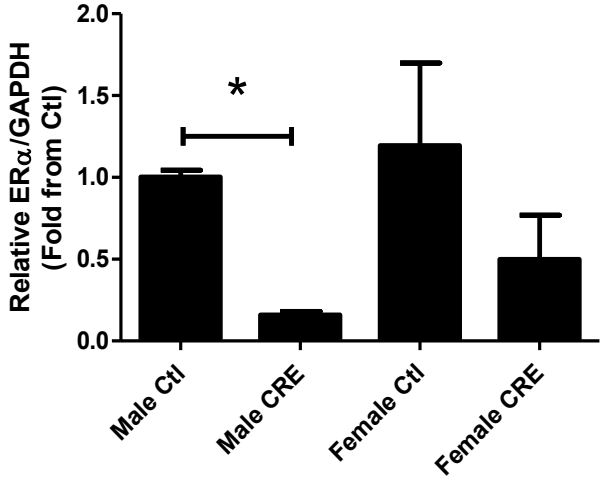


Figure 5. Quantitative real-time PCR showing relative expression of the estrogen receptor alpha (*ESR1* or *ERα*) and *Kiss1* in the liver following hepatic knockdown of *ERα* in homozygous floxed strains with the adeno-associated virus vector AAV8.TBG.PI.Cre.rBG. In **A)** and **C)** *ERα* expression in the liver is reduced in Cre^+ male and female mice compared to control Cre^- mice ($p<0.05$). Asterisk (*) denotes statistical significance ($p<0.05$). *Kisspeptin* expression is upregulated in Cre^+ mice compared to control mice in males and females. In **B)** simple regression analysis shows downward trend (reduction) of *ERα* mRNA in the liver with a corresponding increase in *Kiss1* ($r^2=0.2675$; $p<0.0279$). '-' and '+' denote Control (Cre-negative) and Experimental (Cre-positive) male and female cohorts, respectively.

To assess the expression of *Kiss1* in the liver under dampened *ERα* expression, we carried out a liver-specific knockdown of *ERα* through a tail vein injection of *Cre* recombinase. We injected either *Cre* adeno-associated virus or vehicle under the control of the liver-specific thyroxine-binding globulin (TBG) promoter (AAV8.TBG.PI.Cre.rBG) into Cre^+ and Cre^- (control) male and female mouse cohorts.

In both male and female mouse cohorts (**Fig. 5a** and **5c**, closed bars), there was reduced expression of *ERα* in Cre^+ mice as compared to control Cre^- mice. This observation was statistically significant for male control versus Cre virus injected mice ($p<0.05$), but not for female experimental versus control groups. We also note a significant increase in *Kiss1* expression in Cre^+ mice as compared to control PBS-injected mice (**Fig. 5a**). Figure 5b shows an inverse correlation of relative *Kiss1* and *ERα* mRNA expression levels, with *ERα* decreasing as Kiss1 is upregulated ($r^2= 0.2675$; $p=0.0279$).

4) Assessment of reproductive phenotype

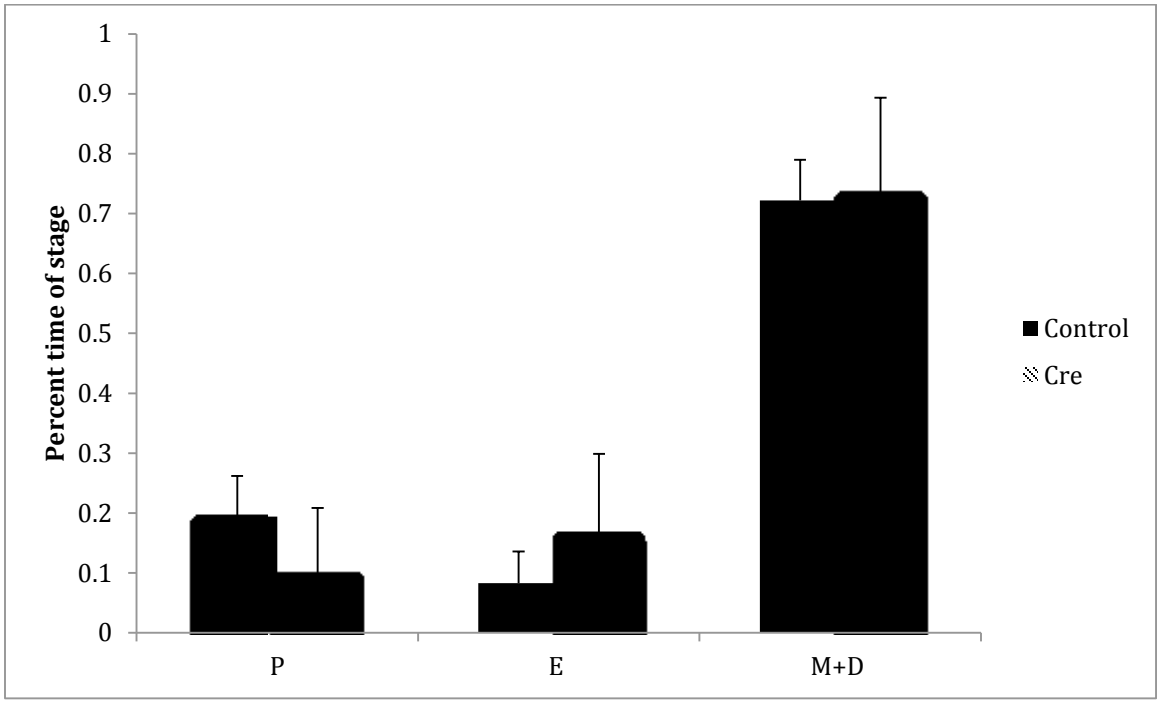


Figure 6. **Estrous cycling of liver-specific ER alpha knockdown mice over a 10-day period**. There was no observed significant difference in percent of time spent in each between control and Cre mice. P=proestrus, E=estrus, M+D= metestrus/diestrus.

The estrous cycles of experimental mice with liver-specific ablations of ERα were compared to those of control mice IP injected with PBS. Estrous cycling was assessed by daily vaginal smear examinations. Stages of the estrous cycle were classified as proestrus, estrus, or metestrus/diestrus based on cytological features outlined in **Table 1**. The percent of time spent in each stage were compared between Cre-injected and control mice. Both groups of mice showed similar frequencies in the amount of time spent in each stage and regular cyclicity, per our criteria ($p>0.05$; not significant). Among the experimental and control mice, the highest percentage of time was spent in the metestrus/diestrus stage characterized by predominant display of leukocytes and nucleated epithelia.

5) Effect of liver-specific knockdown of ER on glucose tolerance

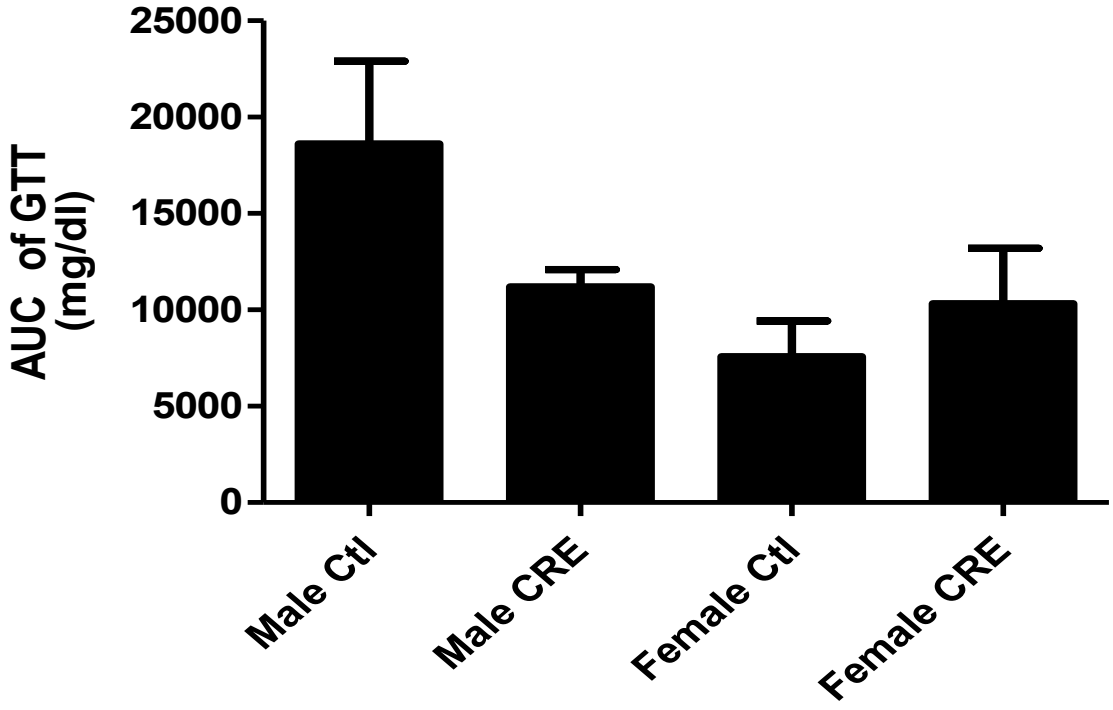


Figure 7. **Area under the curve (AUC) in mg/dl representation of glucose tolerance tests** for male and female AAV.Cre-injected and control saline injected mice. Unpaired T-test showed no significance between male Cre and control, as well as female Cre and control mice groups.

Metabolic phenotype assessment was performed by measuring glucose tolerance after fasting both experimental and control mice overnight (16 hours) and administering bolus IP injection of 2g/kg body weight of dextrose.

Cre -injected male mice showed reduced blood glucose levels compared to saline –injected males, although the difference in reduction was not statistically significant ($p>0.05$). In females, we rather observe a slight increase in glucose levels in experimental versus control mice, although the difference was not statistically significant.

All in all, the knockdown of ERα in the liver did not have any effect on metabolic function, as measured by glucose tolerance in experimental versus control mice.

DISCUSSION

We have demonstrated that in states of acute caloric restriction, *kisspeptin* is robustly upregulated in the liver, while *ERα* expression is significantly reduced in the same state. Under the same conditions, we note that the normal function of the HPG axis is affected as serum gonadotropins LH and FSH significantly reduce. However, when food is reintroduced, *Kiss1* markedly reduces and *ERα* returns to baseline levels. LH and FSH levels also return to normal levels as immediately as 1 hour following the reintroduction of feed.

Further, our data show that knock down of hepatic *ERα* results in increased *Kiss1* expression in both male and female mouse cohorts. It is interesting that male Cre-injected mice exhibit a higher level of knockdown of the hepatic *ERα* than controls, compared to female experimental and control groups. We hypothesize that owing to the greater sensitivity of the female reproductive and metabolic system to the effect of estrogen this observation may be attributable to a more robust compensatory mechanism for estrogen signaling and functioning in females than in males.

Our metabolic phenotype testing analysis shows that, although not significant, Cre virus- injected male mice with knocked down expression of hepatic *ERα* have lower glucose tolerance. In fasted states, glucagon signaling is upregulated (Song *et al*., 2014). Glucagon promotes the nuclear translocation of ERK1/2 and induces the phosphorylation

of the cAMP-response element-binding protein (CREB). Glucagon (induced by low blood sugar levels) regulates the phosphorylation of CREB, a transcription factor crucial for normal pancreatic β cell function and survival. Our laboratory has previously demonstrated that mouse serum from diabetic (db/db) and obese (ob/ob) mice, with high expression of hepatic K*iss1*, could directly impair insulin secretion in pancreatic islets relative to serum from WT mice with the effect being blocked by co-incubation with K234, a KISS1 antagonist (data shown below).

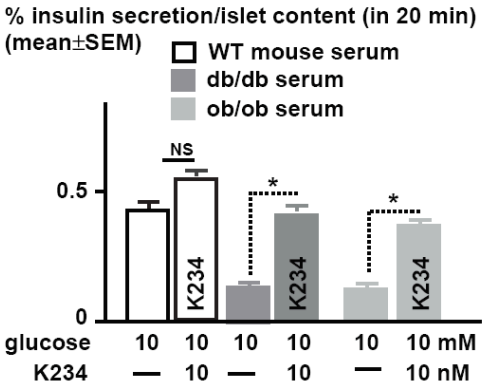


Figure 8. **Serum from mice inhibits insulin secretion.** Serum from WT, db/db or ob/ob mice was added to pancreatic islet preparation at final dilution of 1:10. Insulin secretion measured in absence or presence of KISS1 antagonist (K234). Data are graphed as a % insulin secretion relative to islet insulin content. Serum from mutant mice inhibited insulin secretion, and the effect was blocked by K234. (Hussain *et al*., unpublished).

Insulin is a hormone that is released in response to high glucose concentration. Its functions include the stimulation of the uptake of glucose by myocytes and adipocytes, thereby lowering blood glucose levels. In obesity, glucagon signaling is abnormal resulting in chronic elevated blood glucose levels (hyperglycemia). The underproduction of insulin or insulin insensitivity in obese phenotypes leads to metabolic disorders including type II diabetes.

Reproductive phenotype was assessed following the selective ablation of the estrogen receptor alpha in the liver. The percent of time spent in each estrous cycle stage were compared between Cre-injected and control mice. Both groups of mice showed similar frequencies in the amount of time spent in each stage. Comparing the experimental to control mice, both cohorts spent the highest percentage of time in the metestrus/diestrus stage, which is characterized by a predominant display of leukocytes and nucleated epithelia.

Our findings are novel, as they represent the fundamentals in determining the role of kisspeptin as a signaling link between metabolic status and reproductive response as a function of reduced serum gonadotropins. As already mentioned, although the action of non-hypothalamic kisspeptin on the HPG axis has been unexplored, the pituitary, by virtue of its location outside of the blood brain barrier, is poised to serve as an integrative sensor for peripheral metabolic status, peripheral reproductive status, and central signals regulating reproduction.

Chronic nutritional disturbance impairs the proper functioning of the reproductive neuroendocrine axis. The HPG axis secretes LH and FSH to modulate the maturation, development and function of the gonads, as well as the production of sex steroids. Chronically sub-nourished female rats display a reduction in neuronal *Kiss1* mRNA expression with delayed pubertal onset (as measured by vaginal opening) compared with control rats fed *ad libitum* (Castellano *et al*., 2006). These findings indicate that nutritional deprivation inhibits reproductive function, and as demonstrated by our findings, this inhibition occurs as a result of reduced serum LH and FSH concentrations.

Diet-induced obese mouse models – fed on 60% composition of high fat in their diet (HFD) - exhibit infertility, and previous work from our lab has identified a role for pituitary insulin signaling in the development of neuroendocrine dysfunction and infertility in these obese female mice (Wu *et al*., 2012). We found that HFD-fed mice develop hyperinsulinemia with corresponding infertility and elevated serum LH levels (Brothers *et al*., 2010).

Our preliminary studies suggest that hepatic *Kiss1* expression is acutely regulated in conditions in which metabolic status is associated with reproductive dysfunction. Wolfe and colleagues (unpublished) assessed whether circulating KISS1 could impact central reproductive centers and regulate pituitary LH secretion. In their experiment, LH levels were measured before, and 11 minutes after an IP injection of Kp10 (1nMol) into male and female mice and observed a 3-5 fold increase in serum LH in response to circulating Kp10 (data not shown). This suggests that KISS1 of hepatic origin can regulate hypothalamic and/or pituitary function.

If hepatic *Kiss1* is regulated by metabolic status, and in turn regulates metabolic and reproductive function, it could, therefore, serve as a regulatory hub linking these two important biological systems.

Limitations

Although, in theory, we aimed to achieve a liver-specific knockout with the Cre/LoxP system, our data show an incomplete knockout of the estrogen receptor gene in the liver. This phenomenon may be attributable to technical defects in the delivery of the virus via tail IV injections. Another possible reason is the existence of a rapid turnover

rate of hepatocytes leading to a reduced overall effect of the Cre recombinase on the excision of floxed *ERα* genes.

Our demonstration that caloric restriction significantly reduces hepatic expression of *ERα*, while there is a corresponding robust increase in *Kiss1* expression establishes the basis in answering the larger question of the link between acute changes in metabolic status and reproductive function.

We acknowledge that the signaling link between kisspeptin and E_2 may not be a simple bi-directional interaction between these two molecules. The reproductive system, together with the liver, as a major metabolic organ, are very complex biological systems with multiple players involved in different roles to maintain proper functioning.

For example, the presence of the fibroblast growth factor - 21 (FGF21) metabolic hormone that is predominantly produced in the liver has been identified as one that plays a compensatory role in modulating physiological response to metabolic stress (Woo *et al*., 2013).

Serum FGF21 level increases during fasting due to an upregulation of hepatic peroxisome proliferator-activated receptor- alpha (PPARα) -mediated gene transcription (Inagaki *et al*., 2007; Galman *et al*., 2010). This hormone regulates glucose and lipid metabolism, and mediates glucose metabolism via multiple signaling pathways including the peroxisome proliferator-activated receptor co-activator 1 α (PGC1α) pathway (Potthoff *et al*., 2009). In states of caloric restriction, FGF21 rapidly responds as an endocrine factor to coordinate pleiotropic actions in multiple organs in adaptation to the fasting state (Woo *et al*., 2013).

Owen *et al.* (2013) have demonstrated that FGF21 can impact the HPG axis. In

their study, it was noted that *Kiss1* gene expression in the AVPV of transgenically engineered mouse overexpressing FGF21 was lower compared to wild type mice. Phenotypically, this corresponded to anovulation, and consequently, infertility (Owen *et al.*, 2013).

Additionally, reproductive phenotype assessment was conducted over a period of 10 days following the injection of Cre recombinase to disrupt *ERα* in the liver. The method of classification of estrous cycling from the original work by Nelson *et al*. (1982) was based on a 30-day observation of mice vaginal cytology. The lack of significance in the observed results of our reproductive assessment could be due to the interference of physiological adaptation to stress following the injection of the Cre virus. Not surprisingly, the metestrus/diestrus stage is characterized by an abundance of leukocytes in the vaginal environment, suggesting the upregulation of immunological stress response factors to the injection procedure. Perhaps, in this study, we would have noted significant differences between control and injected mouse estrous cyclicity had reproductive measurements been conducted over a longer time frame. On the other hand, it must be noted that the AAV-Cre vector virus generates a knockout within 3 days of inoculation. Prolonged assessments could therefore introduce confounders into our parameters, since there is the possibility of the hepatocyte regeneration within an extended timeframe.

Taken together, these findings demonstrate that there may be many factors that confound or mediate the outcome of reproductive function in altered metabolic states.

Future Studies

There is the need to experimentally establish whether the *Kiss1* gene is regulated by E_2 in the liver, and, if so, to determine the mechanism of regulation between hepatic *Kiss1* and E_2. Previous studies in the ARC and AVPV reveal that in the presence of estrogen, ERα binds to the promoter region of *Kiss1*, where it induces epigenetic changes leading to the H3 acetylation as well as chromatin loop formation that results in AVPV *Kiss1* expression, while in the Arc the sex hormone exhibits the opposite epigenetic regulation that gives rise to the reduced expression of *Kiss1*.

One way of exploring the epigenetic interaction between ERα and *Kiss1* in the liver relationship is with the use of the chromatin immunoprecipitation (chIP) assay to assess ERα occupancy and changes in repressive and activating histone H3 modifications on the proximal promoter of the *Kiss1* gene. The chIP assay allows for the identification of proteins that are cross-linked to specific regions of the genome through the use of antibodies that bind to the proteins of interest. The method involves the precipitation of the proteins along with the DNA fragment, which is amplified by PCR and, if unknown, sequenced for identification.

Additionally, we plan to clarify the role of hepatic kisspeptin in regulating reproductive function in response to changes in metabolic status by selectively disrupting Kiss1 expression in the liver (L*kiss1* mouse) or by inducing the upregulation of *Kiss1* expression in the liver (CMV-fl-stop-fl-*Kiss1*) with transgenic mouse models. The schematic below (**Fig. 9**) describes the method of generating the *LKiss1* mice. Briefly, it involves the mating of a recombineered mouse with a floxed *Kiss1* allele genotype (i.e. with two loxP sequences flanking the Kiss1 gene) and another with an albumin-Cre

mouse that harbors the Cre recombinase under the control of the liver-specific albumin promoter in its genome. The F1 generation of interest from this mating pair will have hepatic *Kiss1* ablation, while all other tissues will normally express this gene. The production of the CMV-fl-stop-fl-Kiss1 mouse to overexpress hepatic *Kiss1* will require the generation of a mouse model harboring a transgenic cassette expressing mouse kisspeptin cDNA under control of the human CMV promoter. Between the CMV promoter and the kisspeptin cDNA start site codon we will include a floxed stop codon (CMV-fl-stop-fl-*Kiss1* mouse). Thus, the transgene will not produce kisspeptin until removal of the stop codon by Cre recombinase.

Metabolic and reproductive function – measured by *ERα* expression, estrous cyclicity, and fertility - will be tested in these models under normal and challenged metabolic states.

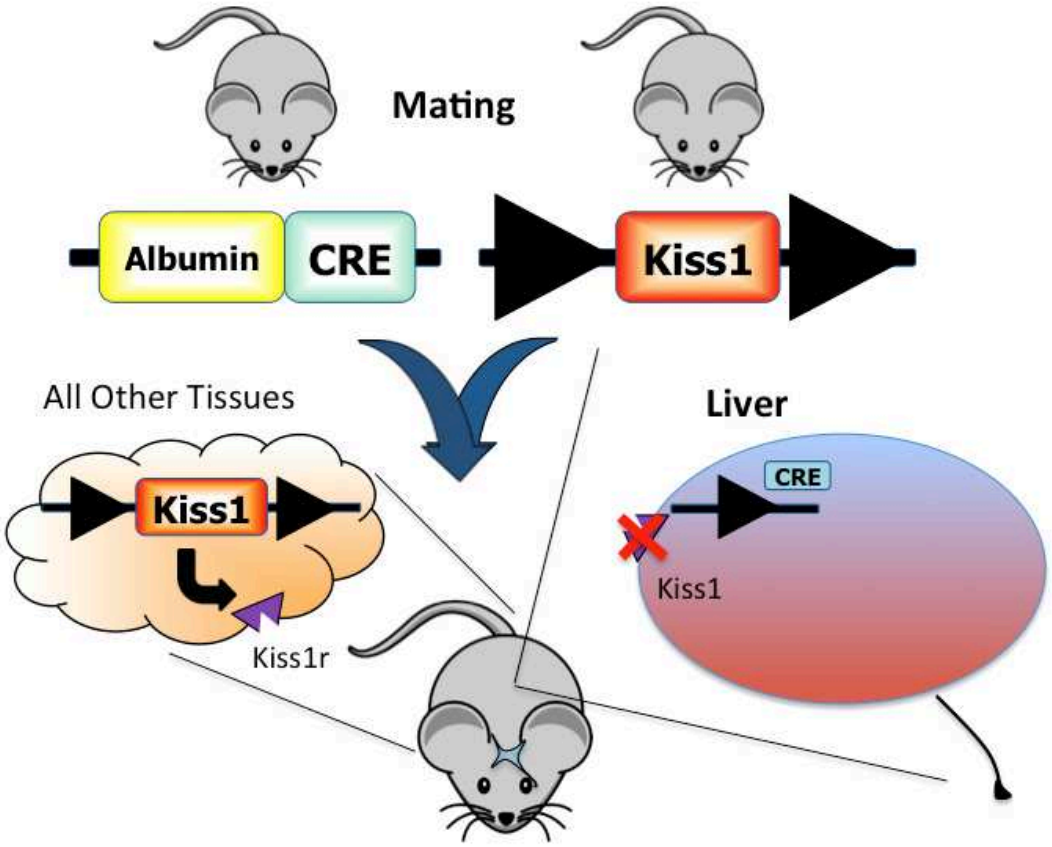


Figure 9. Generation of liver-specific *Kiss1* knockout mice. Mating involving one genetically recombineered mouse bearing Cre recombinase under the control of the liver-specific albumin promoter, and another with floxed *Kiss1* genotype. LoxP sites are represented by dark triangles flanking the *Kiss1* gene. *Kiss1* will be knocked out in offspring liver, while all other tissues will normally express the gene.

Also, insulin tolerance tests will be performed after a 4-hour fast and an intraperitoneal administration of 0.75 units/kg of body weight of insulin. Blood glucose levels will be measured in response to the injection of insulin, as described above, at the same time intervals.

Public health implications

The pathology of polycystic ovary syndrome (PCOS) involves metabolic signaling and reproductive dysfunction. It is the most common endocrine disorder that

affects women of reproductive age, and is most responsible for female infertility (Baldani *et al*., 2014). The most prominent features in the pathophysiology of PCOS pathology include obesity, hyper-production of testosterone, and insulin resistance. Women with PCOS face an elevated risk of other health disorders including metabolic syndrome, cardiovascular disease, and type II diabetes.

Type II diabetes is an endocrinological disease of mounting public health concern. It is also marked by an abnormal metabolic phenotype such as insulin resistance, where the pancreatic β-cells fail to make enough insulin to maintain normal glucose levels. Diabetic complications include cardiovascular disease, kidney failure, lower extremity amputations, visual impairment, and, in extreme cases, fatality arising from hyperglycemic crises (CDC, 2013).

The Centers for Disease Control and Prevention (CDC) reports that between 1980 and 2011, the incidence of type II diabetes in the U.S. population between the ages of 18 and 79 has increased from 498,000 to over 1.5 million (CDC, 2013). These numbers translate to a 133% increase in the crude incidence rate from 3.3 to 7.7 per 1000 population, and an age-adjusted increase in incidence of 117% from 3.5 to 7.6 per 1000 population. The increasing incidence of these endocrine diseases with metabolic impairments has been linked to changes in dietary patterns with the adaptation of high fat diets, and reduced physical activity, due to sedentary lifestyles.

In developing nations, there is an ongoing trend of epidemiological transitioning with the burden of disease shifting from infectious to non-communicable diseases. The World Health Organization (WHO, 2013) recognizes the need to respond to this global

emergency, and, in an effort to control the morbidity and mortality attributable to NCDs, has launched the Global Action Plan (2013-2020) to reduce the incidence of NCDs.

Our study, therefore, contributes to the existing body of knowledge by paving the way for further research to establish the link between metabolic-induced NCDs and their effect on female reproductive health by demonstrating that in mouse models, serum gonadotropin levels are altered by acute changes in metabolic states.

CONCLUSION

We have demonstrated that in states of acute caloric restriction, *kisspeptin* is robustly upregulated in the liver, while *ERα* expression is significantly reduced in the same state. Our findings give evidence to the reduction of serum gonadotropins LH and FSH during acute caloric restriction. Further investigations are necessary to demonstrate the regulation of Kiss1 expression by ERα in the liver during altered metabolic states.

From an energy conservation perspective, it is beneficial for the organism to inhibit reproduction and during states of nutritional deprivation. The results of this thesis, therefore, pave the way for understanding the molecular signaling pathways that coordinate the link between metabolic status and metabolic function.

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SUMMARY OF QUALIFICATIONS

- Solid experience in cellular, molecular and microbiology research
- Ability to converse and write in Spanish fluently
- Utilizing analytical skills to furnish, organize, and interpret scientific data
- Self-motivated, ability to multi-task, and work independently with little supervision
- Communicating professionally and effectively with diverse populations

EDUCATION

Master of Science (2014) – Johns Hopkins School of Public Health, Baltimore, MD
Thesis work - Radovick Laboratory, Johns Hopkins School of Medicine, Dept. of Pediatrics

Bachelor of Arts (2009) – Lawrence University, Appleton, WI, Natural Science Interdisciplinary, Spanish

CERTIFICATES

Global Health (2014) - Johns Hopkins Bloomberg School of Public Health
Maternal and Child Health (2014) – Johns Hopkins Bloomberg School of Public Health
National Board of Public Health Examiners Certification in Public Health (CPH), **NBPHE # 10413**

AWARDS AND HONORS

- Ghana Education Trust Fund Scholarship, Two years' full scholarship; stipend toward graduate education, 2012 – 2014
- Johns Hopkins Bloomberg School of Public Health, Master's Tuition Fellowship, 2013-2014
- Johns Hopkins Bloomberg School of Public Health Student Travel Award, 2013
- Member, Mortar Board National Honor Society, 2008-present
- Dean's List, Lawrence University, 2005-2009

WORK EXPERIENCE

Noguchi Memorial Institute for Medical Research – University of Ghana, Legon
Senior Research Assistant, August 2011- July 2012

- Maintained an ongoing *P. falciparum* culture in human blood medium
- Adept in the preparation of thin and thick blood smears
- Expert in optimizing blood culture to obtain sexual stage parasites
- Maintained a detailed record of experiments in notebooks and software databases
- Spearheaded the course of project with little supervision

Institute for Statistical, Social and Economic Research (ISSER), University of Ghana – Legon, Ghana
Project Assistant, September 2010- 2011 (Part Time)

- Proposed effective governance measures for the reorganized oil and gas industry of Ghana
- Assessed HIV/AIDS spending with UNAIDS and the Ghana AIDS Commission
- Monitored the disbursement of HIV/AIDS funds from the World Bank, USAID, Dutch and Danish governments
- Proposed pilot project to augment scholarly performance among Ghanaian school children
- Contributed to business initiation of the microfinance institution

Lawrence University – Appleton, WI
Admissions Office Student Assistant, September 2005 – December 2007

- Answered prospective students' questions about various aspects of Lawrence University
- Accessed and updated confidential prospective student information
- Built positive representation about student life at Lawrence
- Responded to wellness and safety concerns of prospective parents and guardians
- Proficiently encouraged keen interest in Lawrence in a persuasive manner
- Played a pivotal role in boosting international student enrollment at Lawrence

TEACHING EXPERIENCE
Lawrence University Center for Teaching and Learning (CTL) – Appleton, WI
Chemistry and Statistics Tutor, September 2007- June 2009

- Explained unclear concepts in Inorganic Chemistry and Elementary Statistics to college peers
- Conducted pre-examination group review sessions
- Provided feedback to course instructors and CTL staff on student problem areas in coursework
- Provided recommendations to course instructors on creative ways to foster student interest in course material
- Assisted fellow students with developing effective study strategies
- Facilitated teaching and learning during class sessions

SELECTED PRESENTATIONS AND PAPERS

- Wilson, M.D., Ghansah, A., **Owusu, P.N.**, et al. (2014) *The circadian periodicity of Plasmodium falciparum gametocytes in human peripheral blood and the characterization of biogenic chemicals in malaria transmission* (In preparation)
- de Souza, D., Wilson, M.D., **Owusu, P.N**. (2012). *Impact of climate change on the geographic scope of diseases*. Human and Social Dimensions of Climate Change. ISBN 978-953-51-0847-4, DOI: 10.5772/3242
- Asante, F.A., **Owusu, P.N.** (2010). *Oil and Ghana's development: some global lessons in governance and institution building.* (Unpublished)

- Presented biology research poster at Pew Midstates Consortium at the University of Chicago (2008)

EXTRACURRICULAR ACTIVITIES

- Publication of historical fiction novel *Kwame* for 5-7 year-old children (ISBN-10: B007MDFLOS)
- Marketing and promotion of self-published novel through social media and in-person networking
- Editor of *SEAM*, Johns Hopkins Medical Institutions student literary magazine
- Coordinating and networking in preparation for the "Teddy Bear Hospital" organized by the Child Health Society of the Johns Hopkins School of Public Health
- Served as Lawrence University Welcome Week Leader for two consecutive years (2007-2008)

FOREIGN LANGUAGES

Proficient in Spanish; Ghanaian languages

TECHNOLOGICAL SKILLS

Advanced level MS Excel, PowerPoint, Word, Outlook; STATA, MacOS, Adobe PhotoShop, FileMaker Pro